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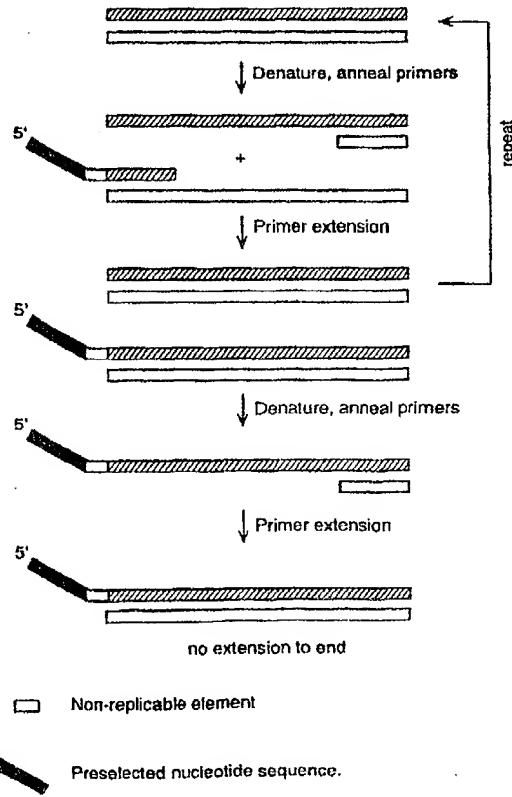
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## (54) Title: NUCLEIC ACID SEQUENCES HAVING SINGLE STRANDED ENDS

## (57) Abstract

The present invention is directed to a method for capturing nucleic acid sequences produced by primer extension reactions. More specifically, the invention relates to a method for producing primer extension products with a single stranded DNA portion suitable for hybridization to a preselected nucleic acid sequence. The invention is useful for the detection of specific nucleic acid sequences which may be present in a sample composed of three portions, the 3' portion is a primer specific for the desired nucleic acid sequence, the 5' portion is complementary to preselected nucleic acid sequence and a portion interposed between the 3' portion and the 5' portion is composed of an element which is not a template for a polymerase. The products of amplification reactions utilizing such a primer have single stranded 5' ends created by the failure of DNA polymerase to "read-through" the primer sequence.



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NUCLEIC ACID SEQUENCES  
HAVING SINGLE STRANDED ENDS

This invention was made with government support under Grant No. HG00099 awarded by the National Institutes of Health. The United States government has certain rights in this invention.

FIELD OF INVENTION

The present invention relates to nucleic acid sequences having single stranded ends and to a method for producing and detecting such nucleic acids. More specifically, the invention relates to extension products of a three element oligonucleotide primer. The 3' element of the primer is specific for replication of the desired nucleic acid sequence. The 5' element is complementary to a preselected nucleic acid probe sequence. The interposed third element is polymerase non-replicable. The products of extension and amplification reactions utilizing such a primer have single stranded 5' "tails" because the DNA polymerase does not "read-through" the interposed third element of the primer sequence.

BACKGROUND OF THE INVENTION

The genome of an organism is unique. Not only do the genomes of different species differ, but the genomes of different individuals within a species differ (with the exception of identical twins or clones). These differences provide individual and species-specific characteristics which can be identified by biochemical techniques dependent upon precise nucleic acid base pairing techniques such as hybridization as well as ligase and polymerase mediated reactions.

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The goal of nucleic acid based diagnostics is the detection of specific nucleic acid sequences. This goal often requires the detection of a specific sequence in the presence of other sequences. In certain cases, it is necessary to discriminate between closely related sequences, even sequences which differ by only a single nucleotide. Methods for doing so are described in various publications. For example, the use of allele-specific oligonucleotide (ASO) hybridization probes for the detection of specific nucleic acid sequences has been described (Wu et al., DNA 8:135-142 (1989); Thein, et al., Br. J. Haematol. 70:225-231 (1988); Conner, et al., Natl. Acad. Sci. USA 80:278-282 (1983); Studencki, et al., Am. J. Hum. Genet. 37:42-51 (1985); Pirastu, et al., N. Engl. J. Med. 309:284-287 (1983); Thein, et al., In K.E. Davies (ed.), Human genetic diseases: A practical approach. IRL Press, Oxford (1986)). The ASO approach allows the discrimination between nucleic acids which differ by as little as a single nucleotide (e.g., alleles). Individual hybridization reactions are required for each allele to be detected. Erlich, et al., Eur. J. Immunogenet. 18:33-55 (1991) and Zhang, et al., Nucleic Acids Res. 19:3929-3933 (1991) have recently described the use of a set of ASO probes immobilized on a membrane and hybridized with labeled polymerase chain reaction (PCR) products. Under appropriate conditions, hybridization is allele specific. Each hybridization can analyze a single amplification reaction.

The present invention permits the detection of specific sequences in a sample. Because a template specific step and the detection step are each controlled by specific but independent base pairing

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requirements, the overall process allows simultaneous detection of multiple templates and multiple samples.

The concept of in vitro DNA amplification was first proposed by Khorana and coworkers in 1971 (Kleppe, K., et al., J. Mol. Biol. 56:341-361 (1971)). Realizing that total chemical synthesis of a gene would result in a finite amount of product, a procedure for in vitro replication was proposed. Their procedure was based on extensive studies of the repair replication reaction, the in vitro replication of a DNA template using a complementary primer (Kleppe, supra (1971)). Their proposal was as follows: "The DNA duplex would be denatured to form single strands. This denaturation steps would be carried out in the presence of a sufficiently large excess of the two appropriate primers. Upon cooling, one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed with the primer. DNA polymerase will be added to complete the process of repair replication. Two molecules of the original duplex should result. The whole cycle could be repeated, there being added every time a fresh does of the enzyme." More recently, this in vitro amplification process has been developed into the polymerase chain reaction (PCR) (Mullis, K., et al. Cold Spring Harbor Symposium Quant. Biol. 51:263-273 (1986); Saiki, et al., Science 230:1350-1354 (1985); U.S. Patent 4,683,202).

Although template amplification improves detection of a particular sequence because a larger amount of template is available for analysis, additional steps are often required after the amplification step for the detection of specific sequences within the amplified product. For example,

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ASO hybridization has been combined with PCR amplification for the specific detection of various disease alleles (Impraim, et al., Biochem. Biophys. Res. Commun. 142:710-716 (1987); Saiki, et al., Nature 324:163-166 (1986); Farr, et al., Proc. Natl. Acad. Sci. USA 85:1629-1633 (1988); Saiki, et al., N. Engl. J. Med. 319:537-541 (1988); Chehab, et al., Nature 329:293-294 (1987)).

The present invention provides an alternative for the analysis for the presence or absence of specific sequences in PCR amplification products.

#### SUMMARY OF THE INVENTION

This invention provides a method for producing primer extension products with a 5' single stranded DNA portion suitable for hybridization to a preselected nucleic acid sequence. The invention thus provides a method for determining whether a particular nucleic acid sequence is present in a sample.

The method for the invention entails the use of a novel three element primer. The 3' element of the primer is complementary to a portion of a template adjacent a target sequence. The 5' element of the primer is complementary to a different preselected nucleic acid sequence. The element interposed between the 3' and the 5' primer elements can not be copied by DNA polymerase. The products of amplification have 5' "tails" which are created by the failure of DNA polymerase to "read-through" the third element primer sequence.

Primers of the invention may be extended on one or both of the templates formed by denaturing a DNA sequence. When extended on both of such templates, a PCR amplification with two single stranded 5' ends results.

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The invention also provides methods for detecting the presence of specific nucleic acid sequences suspected of being present in a sample by the use of probes complementary to the preselected 5' end portion sequences of the primers. Another aspect of the invention includes diagnostic kits applicable to various sequence specific diseases.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic representation of the design of the novel primers of the invention. In the present invention the 3' end of the three element oligonucleotide primer acts as a specific primer for primer extension on a target template. The length of this primer is not critical for the practice of this invention, other than it must be capable of specific priming. U.S. patent 4,683,195 explains that PCR primers "are selected so as to be substantially complementary to each strand of each specific sequence such that the extension product synthesized from one primer, when it is separated from its complement, can serve as a template for synthesis of the extension product of the other primer". Lengths of 15 to 25 are often used for this purpose and in the specific example described, the length of 20 nucleotides was used.

Figure 2 is a schematic representation of the use of the non-replicable element containing primers of Figure 1 in a polymerase chain reaction (PCR). The products contain single stranded 5' ends because of the failure of DNA polymerase to replicate the template past the non-base residues. The product can be hybridized without denaturation to a solid support containing an immobilized pre-selected nucleotide sequence.

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Figure 3 is a schematic of the use of non-replicable element primers to extend both of the templates formed by denaturing a DNA sequence. The extension product has two single stranded 5' ends.

Figure 4 depicts the effect of 1,3 propane diol and 1,4-anhydro-2-deoxy-D-ribitol on primer extension. Templates were synthesized with 1 (P4 and P6, Table 1) or 3 non-base residues (P5 and P7, Table 1) and subjected to a primer extension reaction with three DNA polymerases, Thermus aquaticus DNA polymerase (Taq Pol), DNA polymerase I, Klenow fragment (Klenow Pol) and T7 DNA polymerase (T7 Pol). Controls included the same templates without the non-base residues (P3, Table 1) and a shorter template with a 5' end at the position of insertion of the non-base residues (P1, Table 1).

Figure 5 depicts the hybridization of a PCR product produced with a primer containing non-base residues. PCR reactions were performed with (+) or without (-) 100 ng of human genomic DNA using primer combinations shown. The products of the reaction were hybridized, without denaturation, to a Biodyne C filter containing F02 and F08. The 5' ends of P3 and P5 are complementary to F08 but not F02.

#### DEFINITIONS

As used herein the following expressions have the meanings set forth:

"Oligonucleotide"--refers to a nucleic acid sequence composed of two or more nucleotides. An oligonucleotide can be derived from natural sources but is often synthesized chemically. It can be of any length. It is generally used as a primer, a probe or a component of a ligation reaction.

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"Primer"--refers to an oligonucleotide which is used to initiate nucleic acid synthesis by a template dependent polymerase such as a DNA polymerase. The primer is complementary to a portion of a template nucleic acid.

"Template"--a nucleic acid containing a region sufficiently complementary to a primer such that the primer can anneal and a polymerase can copy the template to produce a complementary nucleic acid.

"Template dependent polymerase"--an enzyme that extends a primer annealed to a template and copies the template to produce a complementary nucleic acid.

"Primer extension"--refers to the process of elongation of a primer on a nucleic acid template. Using appropriate buffers, pH, salts and nucleoside triphosphates, a template dependent polymerase such as a DNA polymerase incorporates a nucleotide complementary to the template strand on the 3' end of a primer which is annealed to a template. The polymerase will thus synthesize a faithful complementary copy of the template. If only a single nucleoside triphosphate is present in a primer extension reaction, then that nucleotide will be incorporated in the primer extension product only if the base of the nucleoside triphosphate is complementary to the base of the template immediately adjacent to the 3' end of the primer.

"Allele"--refers to one of two or more alternative forms of a gene occupying corresponding sites (loci) on homologous chromosomes. The DNA sequence of alleles of a locus differ from each other by at least one nucleotide.

"Non-replicable"--refers to the inability of a polymerase to use the particular portion of a nucleic acid as a template and thus does not replicate it.

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Primer extension is thus arrested at a non-replicable portion of a template.

DETAILED DESCRIPTION OF THE INVENTION

The sample to be analyzed by the present invention may or may not contain the nucleic acid to be detected or discriminated. The nucleic acid may be DNA or RNA, single or double stranded and present in a relatively pure form in the sample or one component of a mixture in the sample. In the case of RNA it is often useful to convert the RNA into DNA using a reverse transcription step. The DNA product can then be analyzed directly or subjected to an amplification step prior to analysis by the present invention.

DNA amplification reactions using in vitro DNA polymerase reactions such as those described by Kleppe, K., et al., J. Mol. Biol. 56:341-361 (1971) and Saiki, et al., Science 239:487-491 (1988) produce DNA which is blunt ended. The double stranded products must be denatured, restriction enzyme digested, or in some other way manipulated to circumvent the problem of blunt endedness.

The method described herein avoids this limitation to the products of amplification reactions by providing a non-replicable end to one or both amplification primers. By interposing between the 3' and 5' ends of an appropriate oligonucleotide primer, non-replicable elements which are not copied by DNA polymerase, the products of amplification have "tails" which are created by the failure of DNA polymerase to "read-through" the primer sequence.

Various non-natural residues have been incorporated into synthetic DNA, including models for the abasic site, a naturally occurring lesion in DNA. Two such residues that maintain the

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inter-atomic distances between the 5' and 3' oxygen atoms of the deoxyribose are 1,4-anhydro-2-deoxy-D-ribitol (Eritja, et al., Nucleosides & Nucleotides 6:803-814 (1987) and 1,3-propanediol (Seela, et al., Nucleic Acids Res. 15:3113-3129 (1987)). The ribitol moiety maintains the same geometric constraints as a true abasic site but has a carbon hydrogen bond at the 1' position instead of a carbon-hydroxyl bond. The presence of a carbon-hydrogen bond at the 1' position of the ribitol renders this moiety stable under conditions that causes hydrolysis of a natural abasic site. Propanediol also maintains the same atomic distance between the 5' and 3' oxygen atoms as a deoxyribose, while allowing for complete freedom of rotation about the intervening carbon-carbon bonds. Both the propane diol and ribitol maintain the distance between the 3' and 5' phosphate linkage in the DNA backbone but lack the nucleosidic base which could stabilize duplex DNA by both base pairing and base stacking.

An abasic site on a DNA template inhibits the chain elongation by a DNA polymerase (Lockhart, et al., Chem. Biol. Interact., 42:85-95 (1982); Schaaper, et al., Proc. Natl. Acad. Sci. USA 80:487-491 (1983)). Similarly, the abasic site analogs severely slow down the incorporation of nucleoside triphosphates in the presence of DNA polymerase (Randall, et al., J. Biol. Chem. 262:6864-6870 (1987)) when present in a template DNA.

To exemplify this invention, DNA polymerase oligonucleotides containing the non-base analogs, 1,3 propane diol and 1,4-anhydro-2-deoxy-D-ribitol were synthesized and used as primers for the PCR.

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EXEMPLIFICATION OF THE INVENTION

Primer design

In the PCR, the product of a primer extension is a template for primer extension in a subsequent cycle. The primer, therefore, becomes a template. If the primer includes a non-base residue, then the strand created with this primer will not be copied completely to the end, but rather will have chain elongation halted at the site of the non-base residue. The PCR primers of this invention were designed with three regions, a 3' region complementary to the desired template, a 5' region complementary to a pre-selected nucleotide sequence and a region interposed between these two containing 1 to 3 non-base residues. This design is shown in Figure 1.

Synthesis of Oligonucleotides

Primer oligonucleotides were synthesized with and without the incorporation of the two non-base residues 1,3 propane diol and 1,4-anhydro-2-deoxy-D-ribitol as described previously (Ugozzoli, et al. GATA 9:107-112 (1992)). The sequences of these primers P1 to P8 are given in Table 1 wherein the "N" in SEQ ID NOS. 4, 5, 9 and 10 symbolizes the 1,3 propane diol and site and "N" in SEQ ID NOS. 6 and 7 the 1,4-anhydro-2-deoxy-D-ribitol site.

Table 1

Name	Length	Sequence 5' ____>3'
<u>Primers</u>		
P1	19	GGGCTGGGCATAAAAGTCA (SEQ ID NO. 1)
P2	12	TGACTTTTATGC (SEQ ID NO. 2)
P3	39	GCACATTGTGCCTGTGACAAGGGCTGGC ATAAAAGTCA (SEQ ID NO. 3)

P1	19	GGGCTGGGCATAAAAGTCA (SEQ ID NO. 1)
P2	12	TGACTTTTATGC (SEQ ID NO. 2)
P3	39	GCACATTGTGCCTGTGACAAGGGCTGGC ATAAAAGTCA (SEQ ID NO. 3)

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P4	40	GCACATTGTGCCTGTGACAANGGGCTGGG CATAAAAGTCA (SEQ ID NO. 4)
P5	42	GCACATTGTGCCTGTGACAANNNGGGCTG GGCATAAAAGTCA (SEQ ID NO. 5)
P6	40	GCACATTGTGCCTGTGACAANGGGCTGGC ATAAAAGTCA (SEQ ID NO. 6)
P7	42	GCACATTGTGCCTGTGACAANNNGGGCTGG GCATAAAAGTCA (SEQ ID NO. 7)
P8	19	AATAGACCAATAGGCAGAG (SEQ ID NO. 8)

Primer extension of oligonucleotides using different enzymes

Primer extension reactions involving an oligonucleotide template and a primer having a sequence complementary to the 3' end of the template were conducted. The primer was extended on the template using *Thermus aquaticus* DNA polymerase, DNA polymerase I (Klenow fragment) or T7 DNA polymerase (Sequenase). Primer extension was monitored by using 5' [<sup>32</sup>P] labeled primers.

Primer P2 was labeled at the 5' end with  $\gamma$ -[<sup>32</sup>P]ATP in a 20  $\mu$ l reaction containing 20 pmol of P2, 40 pmol  $\gamma$ -[<sup>32</sup>P]ATP (>6000 Ci/mmol, DuPont), 50 mM Tris-HCl, pH 7.6, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, and 20 units T4 polynucleotide kinase. Reactions were incubated at 37°C for 1 hours, and stopped by heating to 65°C for 15 minutes.

Primer extension reactions were performed in a total volume of 25  $\mu$ l. Separate reactions contained 25  $\mu$ M of dNTPs (each of dATP, dGTP, dCTP and TTP), 1 pmol each of P1 or P3, P4 or P5 containing 1,3 propane diol, P6 or P7 containing 1,4-anhydro-2-deoxy-D-ribitol as template and 5 pmol of primer P2 (of which 1 pmol was 5'-[<sup>32</sup>P]-labeled). All the reactions were performed in the buffer recommended by the manufacturer of the DNA polymerase. The tubes

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were heated at 92°C for 2 minutes, cooled to room temperature for 15 minutes and then 2.5 units of the respective enzyme were added. All reactions were incubated at 37°C for 15 minutes. 5 µl of each product was mixed with an equal volume of formamide loading buffer, heated to 100°C for 1 minute and then subjected to electrophoresis on a 12% denaturing polyacrylamide gel at 45 Watts. 5'-[<sup>32</sup>P]-labeled P2, P1, P3, P4, P6, P5, P7 and P8 were included on the gel markers (not shown). After the electrophoresis, the gel was exposed to film for 30 minutes.

The results of the primer extension reactions for the various templates are shown in Figure 4. Premature termination of primer extension is clearly seen for all templates containing 1 or 3 of either non-base residue. Lanes 1 to 6 were with enzyme Taq Polymerase, lanes 7 to 12 were with enzyme Klenow and lanes 13 to 18 were with enzyme Sequenase. This experiment demonstrates that the primer extension does not take place beyond either 1 or 3 non-base sites present in a template.

Preparation of filters containing immobilized oligonucleotides

3' amino oligonucleotides F02 (CTTAAACGAAAGCTGCGGTCTNNN (SEQ ID NO. 9)) and F08 (TTGTCACAGGCACAATGTGCNNN (SEQ ID NO. 10)) in which N is 1,3 propane diol were immobilized on Biodyne-C membranes using a modification of the method of Zhang, et al., Nucleic Acids Res. 19:3929-3933 (1991) as described previously (Ugozzoli, et al. GATA 9:107-112 (1992)). 10 pmol oligonucleotides, F02 and F08 suspended in 0.5 M sodium bicarbonate were applied onto the membrane.

Polymerase chain reactions

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Polymerase chain reactions were prepared such that 100  $\mu$ M dNTPs (each of dATP, dCTP, dGTP and TTP) and 10 pmol each of primer 1 and 8, 3 and 8, 5 and 8 respectively in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 0.01% (wt/vol) gelatin in 25  $\mu$ l were below a vapor barrier created using of one Ampliwax PCR Gem (Perkin-Elmer Cetus). The reaction tubes were heated to 80°C for 5 minutes and cooled to 25°C for 1 minute. The total reaction volume for each PCR amplification was 100  $\mu$ l. The reactants above the wax layer were 2.5 units of A-Taq DNA polymerase (Perkin-Elmer Cetus) of 0.2  $\mu$ g of template DNA in PCR buffer. The total volume above the wax layer was always 75  $\mu$ l. PCR was performed in a Ericomp thermal cycler for 30 cycles, each cycle consisting of a denaturation step at 94°C for 1 minute, a primer annealing step at 55°C for 1 minute and an extension step at 72°C for 1 minute. The first denaturation step at 94°C was for 3 minutes and the last extension step at 72°C was for 4 minutes. From each reaction a 10  $\mu$ l aliquot was subjected to electrophoresis in a 1.5% agarose gel. Electrophoresis was performed in 1 X TBE for 2 hours at 150 V. At the completion of electrophoresis the gel was stained in ethidium bromide (0.5  $\mu$ g/ml) for 30 minutes and destained in water for 15 minutes and photographed by ultraviolet transillumination. After viewing the amplified products, a 48  $\mu$ l aliquot of each reaction mixed with 1  $\mu$ l of [ $\alpha$ -<sup>32</sup>P] dCTP (3000 ci/mmol, DuPont) and the reaction heated at 92°C for 2 minutes. After cooling to room temperature for 15 minutes, 2.5 units of Taq polymerase was added and the reaction incubated at 37°C for 15 minutes. An aliquot (10  $\mu$ l) of labeled products was added to 500  $\mu$ L of the hybridizing solution consisting of 5  $\mu$ g/ml Blotto, 6X

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SSPE [1X SSPE = 180 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 8.] 1% SDS, and 10 µg/ml Homomix RNA and the membrane filters were hybridized at 55°C for 2 hours, washed in 6X SSC and exposed to film XAR x-ray film overnight.

Non-base containing primers in PCR

To demonstrate that the non-base residues are useful to produce PCR products with single stranded 5' ends, the oligonucleotide containing three 1,3-propane diol residues was used as a primer to amplify the human B-globin gene by PCR. The normal oligonucleotides (P1 and P3, Table 1) were used as a control. After 30 cycles of amplification, the products of the three reactions, P1 plus P8, P3 plus P8 and P5 plus P8 (as well as no template controls) were subjected to electrophoresis on an agarose gel to determine if amplification was successful. The product of the non-base containing primer was indistinguishable from the products of the normal primers by gel electrophoresis (not shown). In order to determine whether the product of the PCR with the non-base containing primer contained single stranded 5' ends, the products of the reactions were labeled with  $\alpha$ -[<sup>32</sup>P] dCTP in a single cycle of denaturation and primer extension and then the non-denatured products hybridized to filters containing immobilized sequences complementary to the 5' region of the primers. Figure 4 shows the results of the hybridization. The PCR product with P5 plus P8, but not the products with P1 plus P8 or P3 plus P8 hybridized to the oligonucleotide immobilized on the filter. This provides clear evidence that the oligonucleotide containing 1,3 propane diol in the sequence produced a double stranded DNA product with a single stranded 5' end useful for capturing on a

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membrane containing a suitable complementary sequence. The faint background in the P3 plus P8 reaction is likely due to a small amount of single stranded DNA produced due to slight primer ratio imbalance.

The ability to produce double stranded PCR products with single stranded 5' ends has several advantages. By incorporating a pre-selected nucleotide sequence at the 5' end of one of the two primers, the product can be captured by hybridization to a sequence complementary to the pre-selected nucleotide sequence. By using different pre-selected nucleotide sequences on primers for different loci or different alleles, the various amplified products can be produced in a single reaction and detected by hybridization to filters containing sequences complementary to the various pre-selected nucleotide sequences immobilized at defined positions on the filter. The DNA sequence of the pre-selected nucleotide sequence is the choice of the investigator. All sequences in a study could have the same length and GC content providing a single hybridization condition for all sequences.

PCR products having single stranded ends are readily detected. By incorporating a sequence complementary to a labeled probe on the 5' end of one of the primers, a PCR product is produced with a pre-selected nucleotide sequence on one end and a probe complementary sequence on the other. The double stranded molecules with single stranded tails can then be captured and detected using the hybridizability of the tails.

Processes which allow the detection of specific nucleic acid sequences have utility in, among other things, the diagnosis of disease. Thus, for example,

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a process which can allow the detection of the nucleic acid of a pathogen is useful for diagnosing the presence of the pathogen in a biological sample. Furthermore, a process which can discriminate between nucleic acid sequences which are similar, but not identical has utility in diagnosing genetically based diseases. Therefore, commercially available kits which combine the necessary components of a process for detecting a pathogen or a genetically based condition are very important for the diagnosis, treatment and prevention of disease. Accordingly, one aspect of this invention includes kits based on a method for producing and detecting nucleic acid sequences having single stranded ends utilizing a three element oligonucleotide primer to produce primer extension products, wherein the 3' element of the primer is specific for replication of the desired nucleic acid sequence, the 5' element is complementary to a preselected nucleic acid sequence and the interposed third element is polymerase non-replicable. The kits which embody this aspect of the invention contain at least one three element primer, a DNA polymerase and a solid support whereon the complement of the preselected nucleic acid sequence is bound. Kits for amplifying and detecting a specific nucleic acid sequence constant at least two primers, at least one of which is a three element primer containing a first 5' element and the other is labeled or is a second three element primer with a second 5' element suitable for detection with a labeled probe complementary to the second 5' element, a DNA polymerase, a solid support whereon the complement of the first preselected nucleic acid sequence is bound and a labeled probe complementary to the second three element primer. Labels on the

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second primer can include, among other things,  $^{32}\text{P}$ , biotin and fluorescent residues. Labels on the probe can include, among other things  $^{32}\text{P}$ , biotin, fluorophores, alkaline phosphatase, horse radish peroxidase.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: R. Bruce Wallace

(ii) TITLE OF INVENTION: Nucleic Acid  
Sequences Having Single Stranded Ends

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: City of Hope

(B) STREET: 1500 East Duarte Road

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(D) STATE: California

(E) COUNTRY: United States of America

(F) ZIP: 91010-0269

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3M Double Density 5  
1/4" diskette

(B) COMPUTER: Wang PC

(C) OPERATING SYSTEM: MS-DOS (R) Version  
3.30

(D) SOFTWARE: Microsoft (R)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION: Unknown

(vii) PRIOR APPLICATION DATA: None

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Irons, Edward S.

-19-

(B) REGISTRATION NUMBER: 16,541

(C) REFERENCE/DOCKET NUMBER: None

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (202) 785-6938

(B) TELEFAX: (202) 785-5351

(C) TELEX: 440087 LM WSH

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Not known

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE: Not Applicable

(vi) ORIGINAL SOURCE: Synthetically Prepared

(vii) IMMEDIATE SOURCE: Synthetically Prepared

(viii) POSITION IN GENOME: None

(ix) FEATURE: None

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGG CTG GGC ATA AAA GTC A 19

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

-20-

- (A) LENGTH: 12
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Not known
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: No
- (v) FRAGMENT TYPE: Not Applicable
- (vi) ORIGINAL SOURCE: Synthetically Prepared
- (vii) IMMEDIATE SOURCE: Synthetically Prepared
- (viii) POSITION IN GENOME: None
- (ix) FEATURE: None
- (x) PUBLICATION INFORMATION: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TGA CTT TTA TGC 12

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 39
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Not known
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: No
- (v) FRAGMENT TYPE: Not Applicable
- (vi) ORIGINAL SOURCE: Synthetically Prepared

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- (vii) IMMEDIATE SOURCE: Synthetically Prepared
- (viii) POSITION IN GENOME: None
- (ix) FEATURE: None
- (x) PUBLICATION INFORMATION: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCA CAT TGT GCC TGT GAC AAG GGC TGG GCA 30  
TAA AAG TCA 39

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 40
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Not known
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: No
- (v) FRAGMENT TYPE: Not Applicable
- (vi) ORIGINAL SOURCE: Synthetically Prepared
- (vii) IMMEDIATE SOURCE: Synthetically Prepared
- (viii) POSITION IN GENOME: None
- (ix) FEATURE: N represents the moiety 1,3 propane diol
- (x) PUBLICATION INFORMATION: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCA CAT TGT GCC TGT GAC AAN GGG CTG GGC 30  
ATA AAA GTC A 40

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(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Not known

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE: Not Applicable

(vi) ORIGINAL SOURCE: Synthetically Prepared

(vii) IMMEDIATE SOURCE: Synthetically Prepared

(viii) POSITION IN GENOME: None

(ix) FEATURE: N represents the moiety 1,3 propane diol

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCA	CAT	TGT	GCC	TGT	GAC	AAN	NNG	GGC	TGG	30
GCA	TAA	AAG	TCA							42

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Not known

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

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- (iv) ANTI-SENSE: No
- (v) FRAGMENT TYPE: Not Applicable
- (vi) ORIGINAL SOURCE: Synthetically Prepared
- (vii) IMMEDIATE SOURCE: Synthetically Prepared
- (viii) POSITION IN GENOME: None
- (ix) FEATURE: N represents the moiety  
1,4-anhydro-2-deoxy-D-ribitol
- (x) PUBLICATION INFORMATION: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:  
  
GCA CAT TGT GCC TGT GAC AAN GGG CTG GGC      30  
ATA AAA GTC A                                        40

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 42
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Not known
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: No
- (v) FRAGMENT TYPE: Not Applicable
- (vi) ORIGINAL SOURCE: Synthetically Prepared
- (vii) IMMEDIATE SOURCE: Synthetically Prepared
- (viii) POSITION IN GENOME: None
- (ix) FEATURE: N represents the moiety  
1,4-anhydro-2-deoxy-D-ribitol
- (x) PUBLICATION INFORMATION: None

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GCA CAT TGT GCC TGT GAC AAN NNG GGC TGG 30  
GCA TAA AAG TCA 42

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Not known

(ii) MOLECULE TYPE: DNA

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(v) FRAGMENT TYPE: Not Applicable

(vi) ORIGINAL SOURCE: Synthetically Prepared

(vii) IMMEDIATE SOURCE: Synthetically Prepared

(viii) POSITION IN GENOME: None

(ix) FEATURE: None

(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAT AGA CCA ATA GGC AGA G 19

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Not known

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- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: No
- (v) FRAGMENT TYPE: Not Applicable
- (vi) ORIGINAL SOURCE: Synthetically Prepared
- (vii) IMMEDIATE SOURCE: Synthetically Prepared
- (viii) POSITION IN GENOME: None
- (ix) FEATURE: N represents the moiety 1,3 propane diol
- (x) PUBLICATION INFORMATION: None
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTT AAC GAA AGC TGC GGT CTN NN 23

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 23
  - (B) TYPE: Nucleic Acid
  - (C) STRANDEDNESS: Single
  - (D) TOPOLOGY: Not known
- (ii) MOLECULE TYPE: DNA
- (iii) HYPOTHETICAL: No
- (iv) ANTI-SENSE: No
- (v) FRAGMENT TYPE: Not Applicable
- (vi) ORIGINAL SOURCE: Synthetically Prepared
- (vii) IMMEDIATE SOURCE: Synthetically Prepared
- (viii) POSITION IN GENOME: None
- (ix) FEATURE: N represents the moiety 1,3 propane diol

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(x) PUBLICATION INFORMATION: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TTG TCA CAG GCA CAA TGT GCN NN 23

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CLAIMS:

1. A primer having a 3' end portion complementary to a target nucleic acid sequence which may be present in a sample, a 5' portion complementary to a preselected different nucleotide sequence and a non-replicable portion interposed between said 3' portion and said 5' end portion.
2. A primer as defined by claim 1 in which said non-replicable portion is a 1,4-anhydro-2-deoxy-D-ribitol moiety or a 1,3 propane diol moiety.
3. A primer as defined by claim 1 in which said 3' portion of said primer has 15 to 25 nucleotides.
4. A primer as defined by claim 1 in which said 5' portion is complementary to an allele specific probe.
5. A primer as defined by claim 1 in which said 5' portion is complementary to one of a plurality of allele specific probes immobilized on a solid support.
6. A primer as defined by claim 5 in which said solid support is a membrane.
7. A method for detecting the presence or absence of a target nucleic acid sequence in a sample, said method comprising:
  - (i) adding to said sample an oligonucleotide primer to provide extension product including said target sequence, if present, in said sample,  
said primer having  
a 3' end portion complementary to a portion of said target sequence immediately adjacent at least one specific nucleotide to

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provide an extension product of said primer which includes said specific nucleotide,

a 5' end portion complementary to a preselected nucleic acid sequence different from said target sequence, and

a non-replicable portion interposed between the 3' end portion and the 5' end portion;

(ii) adding to the product of step (i) a DNA polymerase to provide for a primer extension product of said primer which includes said target sequence and said specific nucleotide if present;

(iii) subjecting said primer extension product to hybridization conditions with a preselected nucleic acid sequence complementary to the 5' end portion of said primer; and

(iv) determining whether hybridization occurs.

8. A method as defined by claim 8 in which said 3' end portion of said probe has 15 to 25 nucleotides.

9. A method as defined by claim 7 or claim 8 in which said 5' end portion of said primer is complementary to an allele specific probe.

10. A method as defined by claim 7 or claim 8 in which said 3' end portion of said primer is 15 to 25 nucleotides in length, said 5' end portion of said primer is complementary to an allele specific probe immobilized on a solid support, and said non-replicable portion of said primer is a 1,4-anhydro-2-deoxy-D-ribitol moiety or a 1,3 propane diol moiety.

11. In a polymerase chain reaction (PCR) in which a double stranded DNA molecule is denatured to provide two single stranded templates and in which

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each of said templates is replicated by primer extension, the improvement which comprises utilizing a primer as defined by claim 1 or claim 2 to replicate at least one of said templates to produce a PCR product having at least one single stranded 5' end.

12. A PCR as defined by claim 3 in which each of said two templates is replicated utilizing a primer as defined by claim 1 to a PCR product having two single stranded 5' ends.

13. A PCR product having one or two single stranded 5' ends.

14. A duplex PCR product, each strand of which has a single stranded 5' end.

15. A kit for detecting the presence or absence of a target nucleic acid sequence in a sample, comprising

a primer having a 3' portion complementary to said target nucleic acid sequence and immediately adjacent to at least one specific nucleotide of said target nucleic acid sequence to provide an extension product of said primer with a copy of said adjacent nucleotide, a 5' portion complementary to a preselected different nucleotide sequence and a non-replicable portion interposed between said 3' portion and said 5' portion;

a DNA polymerase; and

a solid support having immobilized thereon a nucleic acid sequence complementary to said preselected nucleotide sequence.

16. A kit for amplifying and detecting the presence or absence of a target nucleic acid sequence in a sample, comprising

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at least two primers wherein one of said primers has a 3' portion complementary to said target nucleic acid sequence, a 5' portion complementary to a first preselected nucleotide sequence and a non-replicable portion interposed between said 3' portion and said 5' portion, and another of said primers has a 3' portion complementary to the complementary strand of said target nucleic acid sequence, a 5' portion complementary to a second preselected nucleotide sequence and a non-replicable portion interposed between said 3' portion and said 5' portion, wherein primer extension products of the 3' portion of said primers when synthesized produce nucleic acid sequences that can serve as templates for primer extension reactions utilizing the same primers;

a DNA polymerase;

a solid support having immobilized thereon a nucleic acid sequence complementary to said first preselected nucleotide sequence; and

a labeled nucleic acid complementary to said second preselected nucleotide sequence.

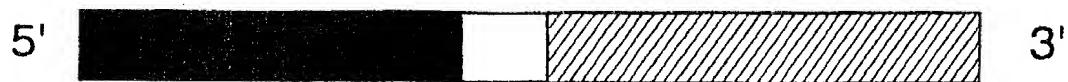
17. A kit as recited in claim 16 wherein the label is chosen from the group consisting of alkaline phosphatase, horse radish peroxidase, a fluorophor biotin or  $^{32}\text{P}$ .

18. A kit useful in performing assays for differentiating a sample containing a target nucleic acid sequence from a sample containing a non-target nucleic acid sequence which comprises multiple containers wherein one of said containers has therein a solution including a primer wherein the 3' element of the primer is specific for replication of the target nucleic acid sequence, the 5' element is complementary to a preselected nucleic acid sequence

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and the interposed third element is polymerase  
non-replicable.

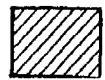
1/5



A preselected nucleotide sequence element > 1 nucleotides in length.



Non-replicable element such as a one or more non-base residues



A nucleotide sequence element > 1 nucleotides in length, preferably > 15 nucleotides in length and complementary to a template.

## FIG. I

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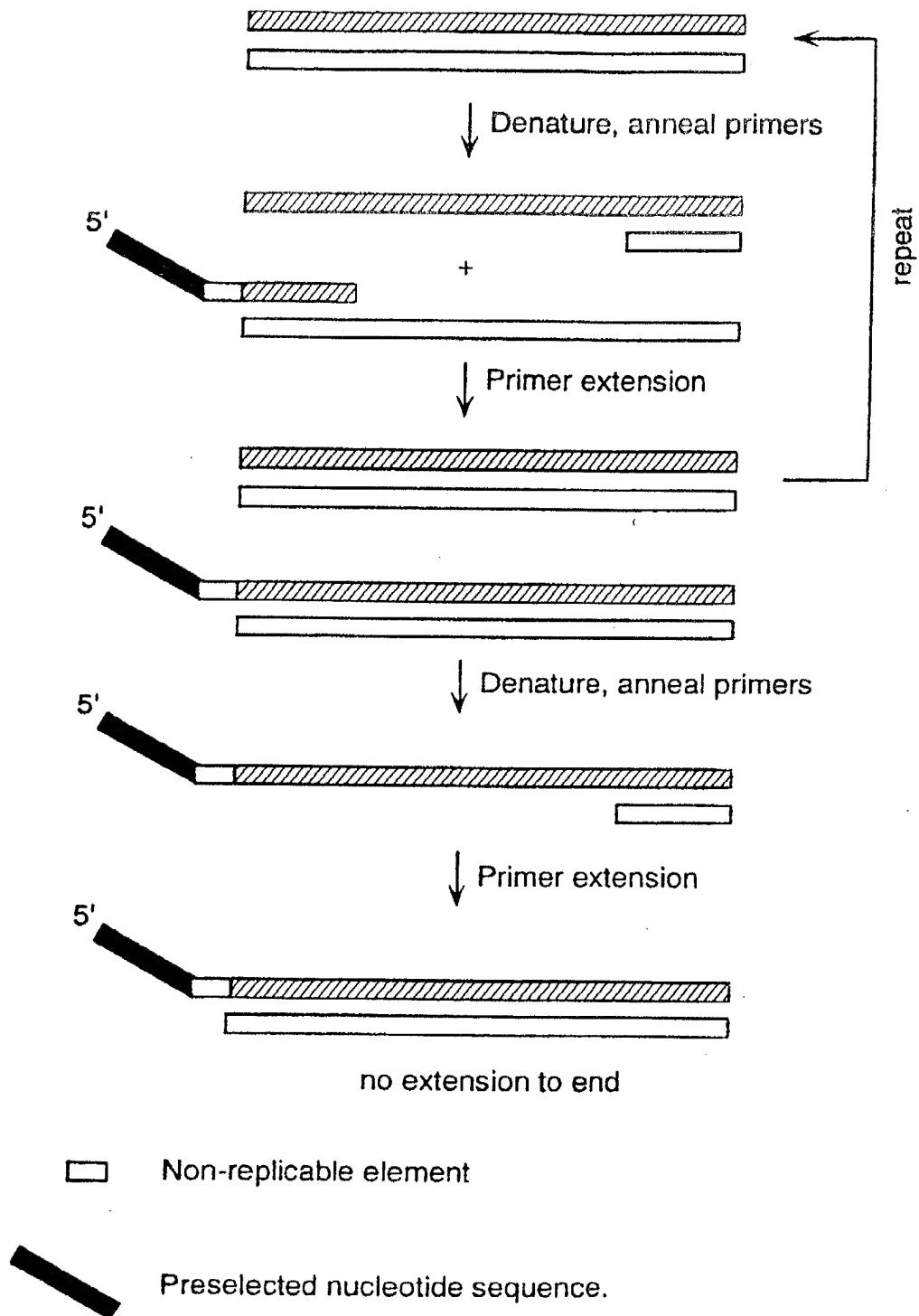
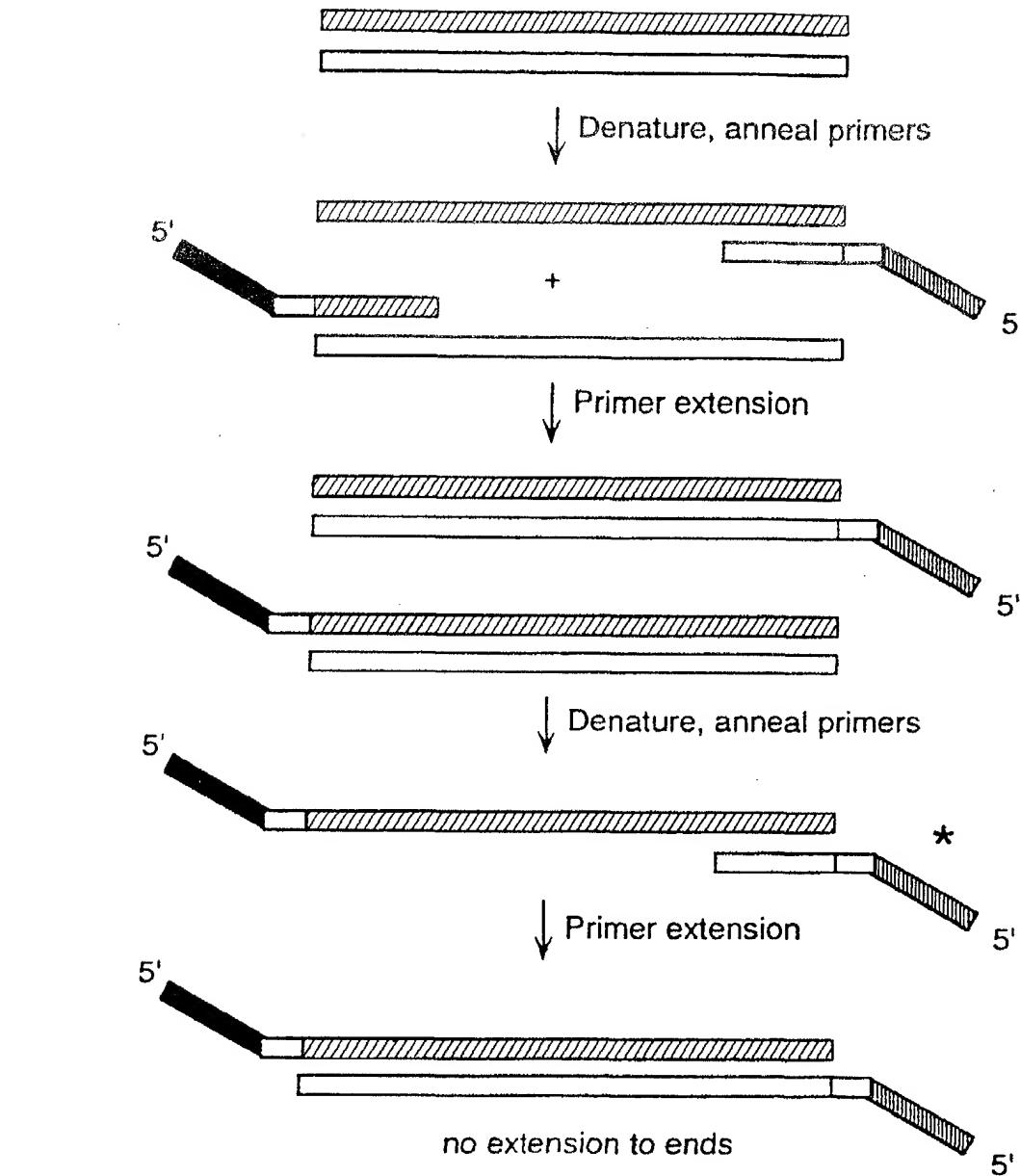


FIG. 2

3 / 5



□ Non-replicable element



Two different preselected nucleotide sequences.

\* Only one of the two primer extension products is shown, the other strand is similarly extended with the other primer to produce the same product.

FIG. 3

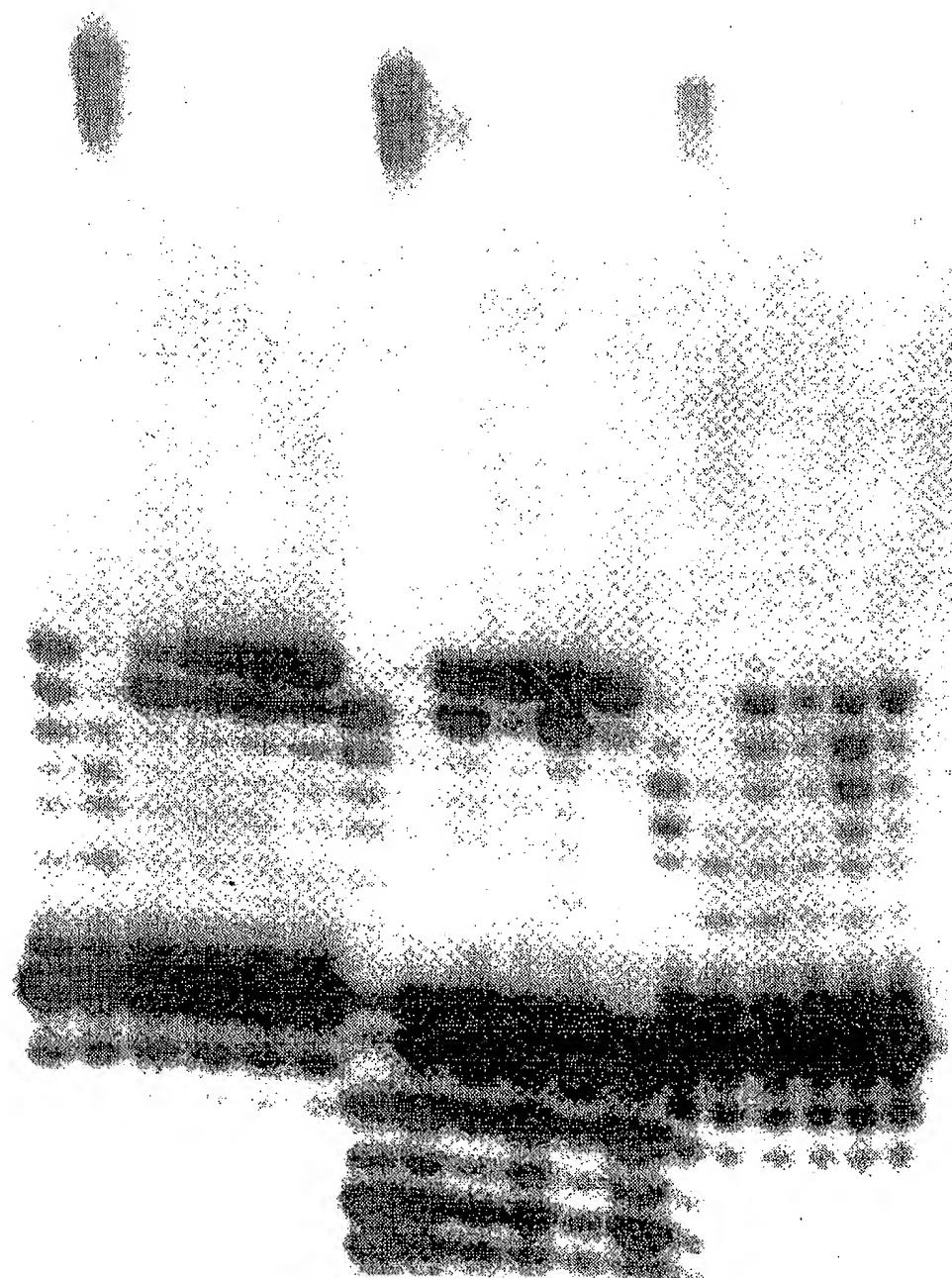
SUBSTITUTE SHEET

183

Taq Pol.

Klenow Pol.

T7 Pol.



**FIG. 4**  
**SUBSTITUTE SHEET**

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IMMOBILIZED SEQUENCE

F02	F08	PRIMERS:	TEMPLATE:
		P5 + P8	*
		P3 + P8	*
		P5 + P8	+
	*	P3 + P8	+
		P1 + P8	+

FIG. 5

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02752

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1/68; C12P 19/34; C07H 15/12  
US CL :435/6, 91; 536/24.3, 24.33

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 536/24.3, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WORLD PATENTS, CA, APS  
search terms: PCR, anchored, capture probe, single-stranded tails, ribitol, propane diol

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Volume 21, No. 5, issued 11 March 1993, C.R. Newton et al, "The Production of PCR Products With 5' Single-Stranded Tails Using Primers that Incorporate Novel Phosphoramidite Intermediates", pages 1155-1162, especially page 1161.	1-18
Y	Methods: A Companion to Methods in Enzymology, Volume 2, No. 1, issued February 1991, E. Loh, "Anchored PCR: Amplification with Single-Sided Specificity", pages 11-19, see entire document.	1-18

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search  
02 JUNE 1993Date of mailing of the international search report  
**14 JUN 1993**Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/02752

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Volume 15, No. 7, issued 1987, F. Seela et al, "Oligodeoxyribonucleotides Containing 1,3-Propanediol as Nucleoside Substitute", pages 3113-3129, especially pages 3114-3119.	1-18
Y	Science, volume 252, issued 21 June 1991, H.A. Erlich et al, "Recent Advances in the Polymerase Chain Reaction", pages 1643-1651, especially pages 1648-1650.	4,5,9,10